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Emerging Microsporidian Infections in Russian HIV-Infected Patients▽

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Microsporidia were identified in stool specimens by histochemistry and PCR of 30 (18.9%) of 159 HIV-infected patients presenting to the S. P. Botkin Memorial Clinical Hospital of Infectious Diseases, St. Petersburg, Russia. The higher prevalence of *Encephalitozoon intestinalis*, in 21 (12.8%) patients, than of *Enterocytozoon bieneusi*, in 2 patients (1.2%), was unexpected. *Encephalitozoon cuniculi* was detected in three patients: one with strain I and two with strain II. *Encephalitozoon hellem* was detected in one patient, and two patients were identified as being infected by *Microsporidium* species. One patient was infected with both *E. intestinalis* and *E. cuniculi*. In two patients, the microsporidian species were not identifiable. No statistically significant differences in gender, age, and stage of AIDS were observed between the microsporidian-positive and -negative HIV-infected patients. HIV-infected patients diagnosed with microsporidian infection, however, were significantly more likely to exhibit ≤100 $CD4^+$ T cells/μl blood (20/30 patients [67%]; odds ratio [OR], 3.150; 95% confidence interval [CI_{95}], 1.280 to 7.750; $P = 0.0116$) and weight loss of >10% of the baseline (19/30 patients [63%]; odds ratio, 2.995; CI_{95}, 1.100 to 8.158; $P = 0.0352$) than HIV-infected patients not diagnosed with microsporidian infection. In summary, this is the first report describing the diagnosis of microsporidian infection of HIV-infected patients in Russia and the first detection of *E. cuniculi* strain II in a human.

The phylum Microsporidia is a group of obligate intracellular, spore-forming, fungal parasites that are ubiquitously distributed among invertebrate and vertebrate hosts. Approximately 1,200 species have been identified, the majority of which parasitize fish and invertebrates. Currently, 14 species of microsporidia are known to infect mammals, including humans (14, 17). Although the first human case of microsporidiosis was reported in 1959 (30), it was not until the AIDS pandemic in the 1980s that microsporidia were frequently recognized as causes of emerging and opportunistic infections of humans. Since then, microsporidiosis in humans has been observed worldwide, mostly in patients with HIV infection and now increasingly in other groups, such as children, immunosuppressed individuals (e.g., organ transplant recipients), contact lens wearers, travelers, and the elderly (17). Affected tissues include muscle, kidney, liver, brain, and cornea, and in AIDS patients, enteric infections seem to predominate, with symptoms of chronic diarrhea, severe weight loss, and malabsorption (24). Among AIDS patients, persistent diarrhea has been attributed to infections with *Cytomegalovirus* and *Cryptosporidium*, followed by the microsporidia *Enterocytozoon bieneusi* and the *Encephalitozoon* species *E. intestinalis*, *E. hellem*, and *E. cuniculi* (37).

HIV infections in Russia have been documented since the late 1980s, and the number of registered HIV-infected individuals grew approximately10,000 to 20,000 annually, to reach a total of 494,000 cases in 2008 (http://hivpolicy.ru/index.php). In St. Petersburg, Russia, the number of HIV-infected intravenous drug abusers increased from 500 in 1999 to 40,000 in 2009 among an estimated population of 5 million citizens (25). Opportunistic infections are major causes of morbidity and mortality in AIDS patients in Russia and worldwide. The frequent occurrence of acute and chronic diarrhea of unknown etiology, especially among HIV-infected patients in Russia with low $CD4^+$ T cell counts in blood, raised concerns that microsporidia may contribute to these clinical signs. Therefore, HIV-infected patients with persistent diarrhea of unknown etiology and/or below-normal $CD4^+$ T cell levels presenting to the S. P. Botkin Memorial Clinical Hospital of Infectious Diseases in St. Petersburg, Russia, were evaluated for the presence of microsporidia in stool specimens by light microscopy (LM) of stained fecal smears as well as by PCR and nucleotide sequencing of amplicons.

MATERIALS AND METHODS

Study subjects and specimens. The study was conducted at the S. P. Botkin Memorial Clinical Hospital of Infectious Diseases in St. Petersburg, Russia, during 2006 to 2009 and was approved by the Ethical Committee (i.e., review board) of the hospital. The patients included men and women ranging in age from 20 to 60 years, most of whom had not received combination antiretroviral therapy (cART), and the HIV/AIDS stage of each patient was classified according to CDC criteria (7). Two hundred forty-seven stool samples collected from 159 HIV-infected patients with chronic and acute diarrhea were evaluated for the presence of microsporidian infection by histochemical staining for light microscopy (LM) or via PCR and nucleotide sequencing of amplicons. On three separate days of patient evaluation, up to 3 stool samples were collected and either stored in 2.5% $K_2Cr_2O_7$ at 4°C or frozen at −20°C until examination. The number of $CD4^+$ T cells in blood was available for 143 patients and was measured within 1 week of the most recent stool sample collection.

LM. Each stool specimen was mixed at a ratio of 1:3 with phosphate-buffered saline (PBS), filtered through a syringe with a gauze plug, and centrifuged at

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TABLE 1. Prevalence of microsporidian species identified in fecal specimens of 159 HIV-infected patients presenting to the S. P. Botkin Memorial Clinical Hospital of Infectious Diseases, St. Petersburg, Russia[e]

Species[b]	No. of patients with positive samples detected by:							Total no. (%) of positive samples
	LM only[a]			LM + PCR			PCR only	
	CW	MTB	Both	CW	MTB	Both		
E. cuniculi							2	2 (1.3)
E. hellem					1			1 (0.63)
E. intestinalis				3	2	9	6	20 (12.6)
E. cuniculi + *E. intestinalis*						1		1 (0.63)
E. bieneusi					1	1		2 (1.3)
Microsporidium sp. 1[c]							1	1 (0.63)
Microsporidium sp. 2[d]							1	1 (0.63)
Undetermined[a]			2					2 (1.3)

[a] Microsporidia detected by light microscopy (LM) but not by PCR were considered undetermined species. CW, calcofluor white stain; MTB, modified trichrome blue stain.
[b] Microsporidian species determination was based on PCR and nucleotide sequencing of rDNA amplicons.
[c] GenBank accession number GQ408913.
[d] GenBank accession number GQ408914.
[e] The total number of positive specimens determined by light microscopy was 2 (1.3%), the total number determined by light microscopy plus PCR was 18 (11.3%), the total number determined by PCR only was 10 (6.1%), and the total number of positive specimens overall was 30 (18.9%).

2,000 × *g* for 2 min. A thin smear was prepared from 20 μl of the pellet suspension, applied onto a glass slide, dried, fixed with methanol, stained with BactiDrop calcofluor white (Remel, Lenexa, KS) for 1 min, washed with tap water, and observed under a Leica DM 2500 microscope equipped with epifluorescence at a magnification of ×1,000. The same slides were then stained with modified trichrome blue stain (Remel, Lenexa, KS) (33) and examined by using bright-field optics.

DNA extraction. DNA was extracted from fecal specimens with minor modifications of a method described previously by Xiao et al. (45). From each specimen, 200 μl of feces was added to a 1.5-ml microcentrifuge tube and washed three times in PBS (pH 7.2) by centrifugation at 10,000 × *g* for 10 min. The pellets were lysed by the addition of 66.6 μl 1 M KOH and 18.6 μl of 1 M dithiothreitol (DTT) and mixed, followed by incubation at 65°C for 15 min. Afterwards, 8.6 μl of 25% HCl, 160 μl of 2 M Tris-HCl (pH 8.3), and 250 μl of a phenol-chloroform-isoamyl alcohol mixture (25:24:1) were added, and each tube was thoroughly mixed. Tubes were then centrifuged at 8,000 × *g* for 5 min, and the upper phase of each sample was transferred into a new tube containing 1.0 ml of ASL buffer from a QIAamp DNA stool minikit (Qiagen, Valencia, CA). Following incubation at 80°C for 5 min, an InhibitEX tablet was added to adsorb PCR inhibitors. Each suspension was incubated for 1 min at room temperature (RT) and centrifuged for 5 min at 20 000 × *g*, after which 200 to 500 μl of supernatant was transferred into a new tube with equal volumes of AL buffer and 100% ethanol. After vortexing, each sample was loaded onto affinity columns, centrifuged for 1 min at 20,000 × *g*, washed twice with AW1 and AW2 buffers, and dried by an additional centrifugation step. DNA was eluted in 100 μl double-distilled water (ddH_2O) and stored at −70°C until applied for PCR.

PCR amplification. Internal transcribed spacer (ITS) and flanking regions of the small subunit (SSU) and large subunit (LSU) of the ribosomal DNA (rDNA) were the targets for the nested-PCR procedure. Upstream primers MSP-1 (TGA ATG [G/T]GT CCC TGT) and MSP-3 (GGA ATT CAC ACC GCC CGT C[A/G][C/T] TAT) target the 3′ region of the SSU and recognized a broad range of microsporidian species, including *Encephalitozoon* spp. and *E. bieneusi* (23). Downstream primers MSP-2B (GTT CAT TCG CAC TAC T) and MSP-4B (CCA AGC TTA TGC TTA AGT CCA GGG AG) target the 5′ region of the LSU of *E. bieneusi*, while MSP-2A (TCA CTC GCC GCT ACT) and MSP-4A (CCA AGC TTA TGC TTA AGT [C/T][A/C]A A[A/G]G GGT) specifically recognize *Encephalitozoon* spp. and some other microsporidia but not *E. bieneusi*. Reactions were performed with 25-μl total volumes using Pure *Taq* Ready-To-Go PCR beads (GE Healthcare, United Kingdom) dissolved in 21 μl of SuperQ water. The first PCR mix contained 1 μl each of primers MSP-1, MSP-2A, and MSP-2B and 1 μl of the template DNA; the second nested reaction included a mixture containing 1 μl each of MSP-3, MSP-4A, and MSP-4B and 1 μl of the first amplification reaction mixture. The expected amplicon size for the MSP-3–MSP-4B (*E. bieneusi*) product was about 500 bp, while that for the MSP-3–MSP-4A (*Encephalitozoon* spp. and other species) product was approximately 300 bp. Amplifications were performed with an Applied Biosystems thermocycler for primary and nested reactions, which included an initial denaturation step at 95°C for 5 min and 36 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 1 min, and elongation at 72°C for 2 min. The final elongation step was extended to 10 min. Positive controls included DNA isolated from *E. intestinalis*-infected RK-13 cells as described previously (15) and *E. bieneusi* from bile samples of necropsied infected rhesus macaques at the Tulane National Primate Research Center (Covington, LA). For negative controls, SuperQ water was substituted for template DNA.

Nucleotide sequencing. Amplicons of the expected (or near-expected) size were excised from the 2% agarose gel and extracted for DNA with the Wizard SV Gel and PCR Cleanup system (Promega, Madison, WI). Sequencing reactions were performed with the Applied Biosystems BigDye Terminator system (version 3.1) and run on a Beckman Coulter Seq 8000 DNA sequencer at the GenLab, School of Veterinary Medicine, Louisiana State University (Baton Rouge, LA). The primers for sequencing were MSP-3, MSP-4A, and MSP-4B, and each amplicon was sequenced in both directions.

PCR analysis of stool samples applied onto FTA cards. Fifty randomly chosen fecal specimens from these patients were applied onto FTA cards (Whatman Inc., Florham Park, NJ). A pea-sized volume was suspended in 1 ml of PBS, filtered through a syringe with a gauze plug, and centrifuged at 14,000 × *g* for 1 min. Each pellet was resuspended in 250 μl PBS and vortexed, and 125 μl of each suspension was applied onto the FTA cards. After drying, the cards were stored at room temperature until use. Discs of 2 mm were cut from the cards, extracted for DNA according the manufacturer's instructions, and subjected to PCR amplification as described above.

Statistical analyses. A Student's *t* test was used to compare means between groups, and Fisher's exact test was used to determine factors associated with microsporidiosis using Graphpad Instat software.

RESULTS

Prevalence of microsporidia in fecal specimens. Fecal specimens from 159 HIV-infected patients were examined, and at least 1 specimen from 30 (18.9%) patients was positive for microsporidia (Table 1). By light microscopy (LM) only, two patients were identified by both calcofluor white and modified trichrome blue staining methods to lend corroboration, but LM did not allow species determinations directly. Among the remaining 28 microsporidian-positive patients that were identified by LM plus PCR or by PCR only followed by sequencing of the amplicons, 2 (1.3%) were identified with *E. cuniculi*, 1 (0.6%) was identified with *E. hellem*, 20 (12.6%) were identified with *E. intestinalis*, 2 (1.3%) were identified with *E. bieneusi*, and 1 (0.6%) was identified with a dual infection of *E. cuniculi* and *E. intestinalis*. Of interest was the detection of 2 (1.3%) patients who were identified as harboring organisms

TABLE 2. Demographic information for the 30 patients with microsporidia of 159 HIV-infected patients presenting to the S. P. Botkin Memorial Clinical Hospital of Infectious Diseases, St. Petersburg, Russia

Patient ID	Age (yr)	Gender[h]	AIDS stage[a]	No. of CD4 T cells/μl blood	Specimen	Species[b]	Histochemistry result[c]	
							CW	MTB
1	26	F	A3	149	1	*E. bieneusi*	+	+
					2	*E. bieneusi*	+	+
					3	*E. bieneusi*	+	+
5	31	M	C3	42	1	*E. cuniculi*	−	−
					2		−	−
16[d]	30	M	B3	140	1	*Microsporidium* sp. 1[e]	−	−
58	27	F	B1	576	1		−	−
					2	*Microsporidium* sp. 2[f]	−	−
63	24	M	C3	4	1	*E. intestinalis*	+	+
					2	*E. intestinalis*	+	+
					3	NT	+	+
66	31	M	C2	450	1		−	−
					2		+	+
69	32	M	C3	47	1	*E. intestinalis*	+	+
					2	*E. intestinalis*	−	−
					3	*E. intestinalis*	−	−
70	45	M	C3	71	1	*E. intestinalis*	−	−
					2	*E. intestinalis*	−	−
					3		+	−
71	29	M	B3	26	1	*E. intestinalis*	−	−
					2		−	−
72	28	F	C3	100	1	*E. intestinalis*	−	−
73	24	F	C3	62	1	*E. intestinalis*	−	−
					2	*E. intestinalis*	+	+
76	36	M	B1	553	1		−	−
					2	*E. intestinalis*	−	−
77	24	F	C3	20	1	*E. intestinalis*	+	+
79	32	M	C2	379	1		+	+
80	33	F	B1	647	1	*E. intestinalis*	−	−
					2	*E. intestinalis*	+	+
81	28	M	B3	26	1	*E. intestinalis*	+	+
					2	*E. intestinalis*	+	+
114	32	M	C3	38	1	*E. hellem*	−	+
118	50	M	C3	11	1		NT	NT
					2	*E. bieneusi*[g]	−	+
124	30	M	C3	92	1	*E. cuniculi*[g]	−	−
137	26	M	C3	60	1	*E. intestinalis*	−	+
138	29	F	B2	463	1	*E. intestinalis*	+	−
139	55	M	C3	21	1	*E. intestinalis*	+	+
140	28	F	C3	180	1	*E. intestinalis*	−	−
141	30	F	C3	68	1	*E. intestinalis*	+	−
142	29	F	C3	31	1	*E. intestinalis*	+	+
					2		+	+
145	35	M	B3	50	1		NT	NT
					2		NT	NT
					3	*E. intestinalis*	−	+
152	29	M	C3	54	1	*E. intestinalis*	+	+
154[d]	57	M	C1	1,213	1	*E. intestinalis*	−	−
156	21	F	C3	49	1	*E. intestinalis* + *E. cuniculi*[g]	−	+
					2		+	+
158	44	F	B3	74	1	*E. intestinalis*	−	−

[a] The stage of AIDS for each patients was based on the CDC scale (7).
[b] Microsporidian species in fecal specimens were identified by PCR and rDNA amplicon nucleotide sequencing. NT, not tested.
[c] CW, calcofluor white, MTB, modified trichrome blue.
[d] Patients who had received cART which was discontinued for more than 1 year at the time of testing for microsporidia.
[e] GenBank accession number GQ408913.
[f] GenBank accession number GQ408914.
[g] Detected by PCR performed on specimens applied onto FTA cards only.
[h] M, male; F, female.

that were placed into the "catch-all" genus, *Microsporidium*, containing as-yet-unclassified species. *E. intestinalis* was also detected in a patient with an unconfirmed HIV status who presented with diarrhea and exhibited a relatively high CD4 cell count of 1,400 $CD4^+$ T cells/μl blood. Of the 30 HIV-positive patients that tested positive for microsporidia by LM and/or PCR, multiple specimens were available for 15 patients (Table 2). Concordant PCR and LM results for multiple spec-

TABLE 3. ITS nucleotide sequence comparisons between microsporidia identified in HIV-infected patient specimens, St. Petersburg, Russia, and GenBank query sequences

Species	Patient ID	Genbank accession no.	Sequence at indicated nucleotide positions of given query sequence							
			143–149	291–299	7641–7644	7801–7804	7809–7912	7813–7816	7817–7820	7821–7824
E. cuniculi		AL391737			TCGC	GGAT	GTTT	GTTT	GTTT	GTGT
	5				***T	****	****	****	****	****
	124				***T	****	****	****	**_ _	****
	156				***T	****	****	****	**_ _	****
E. hellem		AF338367		TAG-CGACG						
	114			**AR*****						
E. intestinalis		Y11611	GAGGATT							
	140	CG408911	******C							
	152	CG408912	*****C*							

imens were reported for three patients (patients 1, 63, and 81), and concordant results with PCR only were detected for the multiple specimens of another three patients (patients 69, 73, and 80). Among the remaining patients for whom multiple specimens could be evaluated, discordant results were observed between specimens, suggesting that the shedding of microsporidia varied from stool sample to stool sample, that diagnostics testing was inconsistent, or that this might represent transient colonization by microsporidia.

Demographic and clinical features. No significant difference in gender or age was observed for the microsporidian-positive versus -negative groups of HIV-infected patients, although there was a preponderance of males (68%) presenting to the clinic overall. The group of 30 patients positive for microsporidia in stool specimens included 12 females (40%) and 18 males (60%), ranging in age from 24 to 57 years (mean, 32.6 years; median, 30 years; 95% confidence interval [CI_{95}], 24 to 55). $CD4^+$ T cell levels ranged from 4 to 1,213 $CD4^+$ T cells/μl blood (mean, 154 T cells/μl blood; median, 68 T cells/μl blood; CI_{95}, 11 to 647), and patients with microsporidia were more likely to exhibit $CD4^+$ T cell counts of ≤100 cells/μl blood at the time of diagnosis (20/30 patients [67%]; odds ratio [OR], 3.150; CI_{95}, 1.280 to 7.750; $P = 0.0116$) and weight loss of >10% of the baseline (19/30 patients [63%]; OR, 2.995; CI_{95}, 1.100 to 8.158; $P = 0.0352$) than were the HIV-infected patients having no microsporidia detected or having used cART. The stage of AIDS at which the patients presented was determined according to the CDC scale and ranged from A1 to C3. Only 2 of the 30 patients who were positive for microsporidia had received cART, and although therapy was discontinued at least 1 year prior to evaluation for microsporidia, one patient (patient 154) continued to exhibit a relatively high CD4 T cell level (Table 2).

rDNA sequencing. Nucleotide sequences of amplicons were obtained to confirm the species identification. The most prevalent microsporidian species, *E. intestinalis*, was detected in 21 HIV-infected patients and 1 patient with unconfirmed HIV with 1,400 $CD4^+$ T cells/μl blood. The amplicon nucleotide sequences from 20 individuals exhibited 100% homology with *E. intestinalis* (GenBank accession number Y11611), whereas amplicons from two patients with *E. intestinalis*, patients 140 and 152, exhibited base pair mismatches at positions 148 and 149 in the ITS region (GenBank accession numbers CG408911 and CG408912, respectively) (Table 3). *E. cuniculi* was identified in three patients, patients 5, 124, and 156 (who carried a mixed infection with *E. intestinalis*). These isolates exhibited 97% homology with the *E. cuniculi* sequence reported under GenBank accession numbers AL391737 and AJ005581. The number of 5′-GTTT-3′ repeats in the ITS region is one of the characteristics used to designate the strain (genotype) of *E. cuniculi* (16). The amplicon from patient 5 exhibited three repeats, indicative of strain I, which was originally isolated from rabbits, and the amplicons from patients 124 and 156 expressed two repeats, indicative of strain II, originally isolated from mice. *E. hellem* was identified in the fecal specimen of patient 114 by PCR, and a blastn comparison of the rDNA amplicon matched with 99% identity to *E. hellem* genotype 1A (GenBank accession number AF338367), with a mismatch of A versus G at position 293 and an insert of R (A/G) at position 294 relative to the query sequence. *E. bieneusi* was detected in specimens from two patients, patients 1 and 118, by PCR, and rDNA sequences were identical to that of the *E. bieneusi* sequence under GenBank accession numbers A4371284, DQ793213, AY237216, AF023245, and AF101200, which correspond to *E. bieneusi* genotype D.

The rDNA sequences of amplicons from two patients, patients 16 and 58, that were similar in size to amplicons produced for *Encephalitozoon* (i.e., approximately 300 bp), displayed relatively low homology to any of the microsporidian rDNA sequences deposited in the GenBank database. A catch-all genus, *Microsporidium*, was established to temporarily place such uncharacterized microsporidia until further studies are performed and reclassification can be established (6). The sequence from *Microsporidium* sp. strain 1 (M1), from patient 16 (GenBank accession number GQ408913), exhibited 82% and 86% homologies (over 84% and 89% coverage) with an *Episetum* sp. and *Larssonia obtusae*, a species infecting *Cladocera*, respectively. The rDNA sequence of *Microsporidium* sp. 2 from patient 58 exhibited 84% (with only 41% coverage) homology with the lepidopteran microsporidian *Vairimorpha* sp.

DISCUSSION

Prior to the implementation of cART in the mid-1990s, the prevalence of microsporidiosis in HIV-infected individuals

worldwide ranged between 5% and 50%, with an overall average of 15% (5). After cART became available in developed countries, the prevalence of opportunistic infections, including microsporidiosis, dramatically declined in relation to improved immune status (4, 17, 18, 20, 21, 31, 40, 43). In developing countries, however, where HIV-infected patients have limited or no access to cART, opportunistic infections, including those due to microsporidia, remain problematic (4, 19, 20). Recent cross-sectional surveys conducted since the year 2000 have continued to report microsporidiosis with prevalence rates of 27.5% in Brazil (3), 5.2% in Cameroon (36), 16% in Ethiopia (19), 11% in Guinea-Bissau (27), 1 to 26.7% in India (10, 26, 41), 10.5% in Niger (20), 42.7% in Portugal (21), 21.6% in South Africa (34), 81.2% in Thailand (44), 11.8 to 20% in Tunisia (1, 8), 77% in HIV-infected children in Uganda (42), 17.4% in Venezuela (9), and 9.5% in Vietnam (20). This report is believed to be the first identification of microsporidian infections in HIV-infected individuals presenting to a major city hospital in Russia. Since relatively few of these individuals used cART, the prevalence of 18.9% (i.e., 30 of 159) for patients with microsporidia in fecal specimens is consistent with reported prevalence rates prior to the implementation of cART. The results of this study were also consistent with previous reports associating microsporidiosis in patients with lower $CD4^+$ T cell levels and weight loss (11, 12).

The most prevalent microsporidian identified in HIV-infected individuals with diarrhea worldwide has been *E. bieneusi* (17), so it was unexpected that *E. intestinalis* was the most frequently detected microsporidian species in this group of patients in Russia. *E. bieneusi* and *E. intestinalis* are generally detected in feces, and *E. intestinalis* then often disseminates to also cause systemic infection (24). The other two *Encephalitozoon* species, *E. cuniculi* and *E. hellem*, are rarely detected in feces, usually cause systemic disease, and are shed primarily in urine rather than feces (17). The detection of *E. cuniculi* in the feces of three patients and *E. hellem* in the feces of another patient raises questions about whether all three *Encephalitozoon* species may contribute to diarrhea and, furthermore, whether these HIV-infected individuals will also exhibit disseminated infections and disease. Further testing of urine for the presence of microsporidiosis in HIV-infected patients may thus be warranted. Also noteworthy was that microsporidian shedding was sometimes inconsistent, such that replicate fecal specimens collected from a given patient were positive on one day but negative on another day. This suggests that several fecal specimens may need to be tested before microsporidia can be ruled out as a cause of diarrhea.

The detection of *E. cuniculi* strain II in an HIV-infected patient was also of interest. One of the markers for genotype identification in this species is the number of 5′-GTTT-3′ repeats in the ITS rDNA genes (16). To date, humans have been reported to have infections due to *E. cuniculi* strain I (initially isolated from a rabbit, with 3 repeats) and strain III (initially isolated from dogs, with 4 repeats) (15). To our knowledge, this is the first report of *E. cuniculi* strain II being identified in humans. *E. cuniculi* infections in humans are considered primarily of zoonotic origin, and *E. cuniculi* strain II was previously isolated in mice, rats, blue foxes, birds, and a captive adult Goeldi monkey (28, 29). St. Petersburg, Russia, is experiencing an increased rat population, which may have contributed to the transmission of this microsporidian to HIV-infected individuals.

E. hellem infection was also likely due to a zoonotic exposure, most probably from birds that are considered natural hosts for this species (22, 38, 39). Conversely, *E. intestinalis* is only rarely detected in animals, so the most likely source of this infection is other infected humans (2, 13). *E. bieneusi* infects a wide range of animals (as well as humans), and the genotype identified in the patients in St. Petersburg, Russia, has also been identified in beavers, cattle, dogs, falcons, foxes, macaques, muskrats, pigs, and raccoons, suggesting that these infections could have occurred via horizontal transmission between humans or via zoonotic reservoirs (35).

The two *Microsporidium* spp. detected by PCR, but not by LM, in two patients with relatively high $CD4^+$ T cell levels suggested a very low parasite burden or perhaps that these species were not infectious (i.e., ingested and passively shed in feces). Alternatively, these microsporidia could be considered new infectious microsporidia in humans if shedding with these species were to continue or reoccur.

The MSP primers (23) used in these studies amplify several microsporidian species that infect primarily humans. In this study, these primers also amplified rDNAs of two unknown microsporidia as well as a few spurious amplicons. Two amplicons with sizes identical to those of amplicons produced by *Encephalitozoon* spp. (i.e., approximately 300 bp) were generated. The nucleotide sequences of these two amplicons were sufficiently related to other microsporidian species but warranted classification into the "catch-all" genus until further examination. Another four amplicons were of sizes similar to those generated for *E. bieneusi* (i.e., 500 bp), but these nucleotide sequences, two amplicons from patient 2 and another amplicon from patient 31, were most related to *Candida albicans* (GenBank accession number L28817). The sequence of a fourth amplicon from patient 80 was most related to *Bacteroides thetaiotaomicron* (GenBank accession number AF0159280). These findings support the importance of including known microsporidian species controls in the PCR amplification and for sequence analyses of amplicons.

PCR cannot always be performed at hospitals or medical centers in the developing world, so 50 randomly selected fecal specimens were applied onto Whatman FTA filter cards for the later performance of DNA extraction, amplification, and nucleotide sequencing (32). Two of the specimens that tested negative by PCR performed directly on the fecal specimens did generate amplicons from DNA extracted from the FTA cards (patients 118 and 124), possibly due to the presence of PCR inhibitors in feces. Conversely, four specimens from which microsporidia were detected by PCR performed directly on fecal specimens (i.e., patients 80, 140, 142, and 156) tested negative by PCR with the FTA cards. Concordant results were obtained between the remaining 44 randomly tested specimens. This further suggests, however, that several consecutive specimens need to be tested before microsporidiosis can be ruled out and that sensitivity levels need to be compared between PCR performed on DNA extracted directly from specimens and PCR performed on DNA from FTA filter cards.

In summary, this study contributes to the recognition of microsporidia as causes of emerging and opportunistic infections now being observed for HIV-infected patients in Russia. The

results differ from those of previous prevalence studies, since *E. intestinalis* was identified more frequently than *E. bieneusi*. This study, for the first time, also identifies *E. cuniculi* strain II isolated from humans. The results also support the repeat testing of sequentially collected patient specimens before microsporidia can be ruled out as an associated cause of diarrhea. Furthermore, continued studies will be important for determining the sources of these infections to help reduce the prevalence and for determining if these microsporidian infections also cause disseminated (systemic) disease in these HIV-infected patients or other immunocompromised individuals in Russia.

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